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Isolation of a Novel *Aggregatibacter actinomycetemcomitans* Serotype b Bacteriophage Capable of Lysing Bacteria within a Biofilm▽†

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A bacteriophage specific for *Aggregatibacter actinomycetemcomitans* serotype b, able to kill the bacterium within a biofilm, was isolated. Random mutagenesis of this phage rendered a bacteriophage able to kill 99% of the bacteria within a biofilm. This is the first report of a biocontrol experiment against *A. actinomycetemcomitans*.

Periodontitis is an infection of the supporting tissues of the tooth caused by bacteria or bacterial groups embedded in a biofilm (14). *Aggregatibacter actinomycetemcomitans* (formerly, *Actinobacillus actinomycetemcomitans*) is a capnophilic, nonmotile Gram-negative bacterium (11, 22) related to the aggressive form of periodontitis (6–10, 18, 23); its isolates are classified into seven serotypes, a to g (12, 24), with serotype b frequently associated with disease (2, 13, 20) and serotype c with oral health (19). Periodontitis caused by *A. actinomycetemcomitans* often requires antibiotic therapy besides mechanical treatment due to the bacterium's ability to form a biofilm in the periodontal pocket and on all mucous membrane surfaces in the oral cavity (6). Biofilms are organized in highly efficient and stable ecosystems (15), and it has been proposed that bacterial susceptibility to antibiotics is reduced within this structure (4), making it virtually impossible to completely remove bacteria from biofilms with antibiotics only (6). These features make periodontitis a complex disease and motivate the search for novel antimicrobial therapies such as oral microbiota modification and phage therapy (1, 16). Phage therapy has been attempted for systemic diseases (1) and to control infections in the oral cavity (17). Our aim was to isolate a bacteriophage for *A. actinomycetemcomitans* and evaluate its effec-

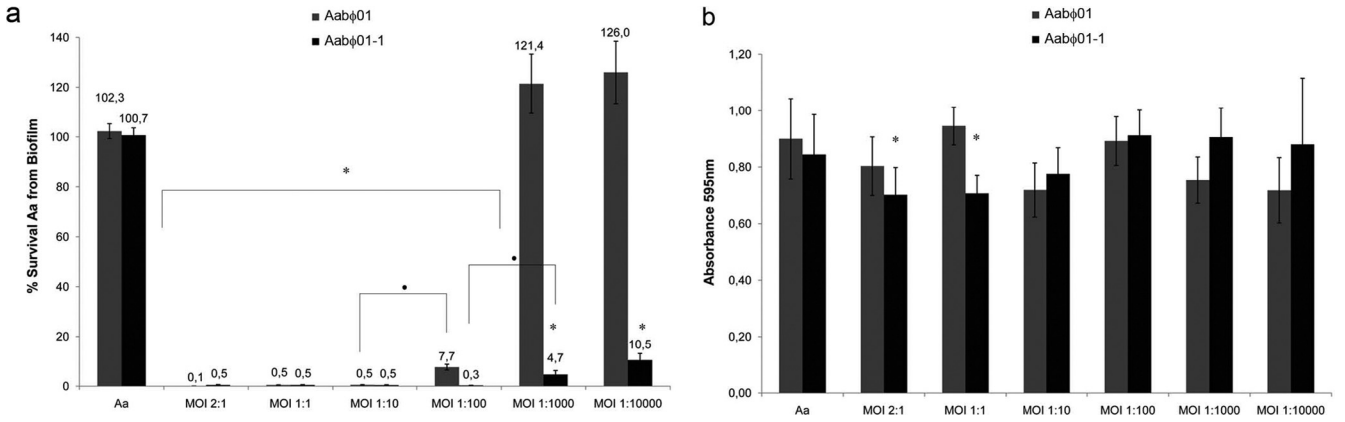

FIG. 1. Infection of an *A. actinomycetemcomitans* biofilm with phages Aabϕ01 and Aabϕ01-1. A biofilm of *A. actinomycetemcomitans* cultured during 24 h under capnophilic conditions was infected with Aabϕ01 or Aabϕ01-1 at different MOIs. The results are expressed as percentages of CFU/ml recovered 24 h postinfection compared with the CFU/ml recovered before infection (a) or from quantified biofilm at 595 nm (b). As a control, we used a biofilm of *A. actinomycetemcomitans* without infection (Aa). Bars depict the average values from three independent experiments. An asterisk represents significance in relation to the control ($P < 0.05$).

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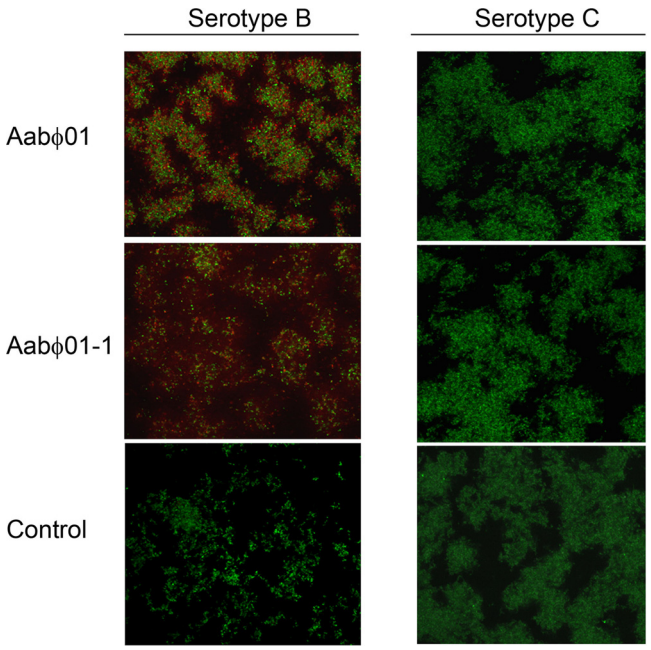

FIG. 2. Confocal microscopy of a biofilm of *A. actinomycetemcomitans* after infection with Aabϕ01 or Aabϕ01-1. A biofilm of *A. actinomycetemcomitans* serotype b or serotype c cultured during 24 h under capnophilic conditions was infected with Aabϕ01 or Aabϕ01-1 and was stained with the LIVE/DEAD BacLight bacterial viability kit. As a control, we used an *A. actinomycetemcomitans* biofilm without infection. Green indicates live bacteria, and red indicates dead or dying bacteria.

tiveness on *A. actinomycetemcomitans* biofilms as a first evaluation of phage therapy for aggressive periodontitis.

Isolation of an *A. actinomycetemcomitans* bacteriophage. The strains used in this work are listed in Table S1 in the supplemental material. Seventeen clinical samples were obtained from saliva and wastewater from dental chair drainages. They were propagated by adding 2 ml of the sample to 20 ml of *A. actinomycetemcomitans* strain PAA005 at an optical density at 600 nm (OD_{600}) of 0.15 to 0.2. After 24 h of incubation at 37°C, supernatants were spot tested over lawns of PAA005 grown on brain heart infusion (BHI) agar. One sample generated a clearing zone; a bacteriophage was isolated from it by diluting and plating for isolated lysis plaques and designated Aabϕ01. Phage Aabϕ01 formed turbid plaques of about 0.5 to 1 mm in diameter (see Fig. S1 in the supplemental material); this could be indicative of a temperate phage, which would not be surprising since they are commonly found for this bacterium (3, 10). To test for the host range, other bacteria were tested, including *A. actinomycetemcomitans* serotype c strains, and were not susceptible to Aabϕ01, indicating that this phage has a restricted host range. To test if PAA005 has an inducible infectious viral particle in its genome, a culture of PAA005 was induced by UV light at 302 nm for 40 s (3), and the supernatant was tested as described above. No bacterial lysis was observed (data not shown), indicating that Aabϕ01 was not a product of a lysogen induction.

Infection of *A. actinomycetemcomitans* by Aabϕ01. Infection of PAA005 liquid cultures at early exponential phase (OD_{600} of 0.1 to 0.2) with Aabϕ01 caused the exponential phase to be interrupted at an OD_{600} of 0.5, the point at which a massive cell lysis, faster than the bacterial generation, started, causing a continuous decrease in the OD_{600} until a stable reading of 0.22, indicating that the phage did not kill the bacterial culture completely. To try and improve the lysis, we performed a mutagenesis on phage Aabϕ01 by exposing a suspension of phage to UV light (260 nm) from a 15-W germicidal lamp at a dose rate corresponding to about 15 min (26). After UV treatment, serial dilutions were made from the lysate and spotted on the surface of a lawn of *A. actinomycetemcomitans*. Clear lysis plaques were selected and propagated, and one new lysate was chosen for further studies; it was designated Aabϕ01-1. When a PAA005 culture at early exponential phase was infected with Aabϕ01-1, the exponential phase reached an OD_{600} of 0.35, and we observed an improvement of the cell lysis with a decrease in the OD_{600} to 0.1.

Antimicrobial activity of phages Aabϕ01 and Aabϕ01-1 on an *A. actinomycetemcomitans* biofilm. An *A. actinomycetemcomitans* biofilm was allowed to form on polystyrene flat-bottom 96-well plates (Cellstar Greiner Bio One, Germany) inoculated with 200 μl of a 1/100 dilution of a PAA005 overnight culture incubated in a capnophilic (CO_2-rich) environment at 37°C. The biofilm present on each well was stained with 200 μl of a 0.01% crystal violet solution for 15 min and quantified by solubilizing the crystal violet with ethanol and measuring its absorbance at 595 nm (5, 21). Additionally a CFU count was performed by plating dilutions on BHI agar. To challenge this biofilm with phages, the culture medium was removed, and fresh culture medium containing phage at different multiplicities of infection (MOIs) was added. At 24 h postinfection, the CFU and the amount of biofilm were measured. Antimicrobial activity of phages on an *A. actinomycetemcomitans* biofilm was analyzed by comparing the CFU/ml of bacteria recovered from 24-h biofilms treated with the phage lysate at different MOIs and those recovered from untreated biofilm. The minimum necessary MOI to achieve the best decrease of the *A. actinomycetemcomitans* counts for the Aabϕ01 phage was 0.1, while for the Aabϕ01-1 phage, it was 0.01 (Fig. 1a). Interestingly, phages were not necessarily able to reduce the amount of biofilm, showing this ability only with the phage Aabϕ01-1 at a high MOI (Fig. 1b). We characterized the cell viability within the biofilm by confocal microscopy. Biofilms formed on coverslips were treated with phages as described above and stained with Syto9 and propidium iodide (LIVE/DEAD BacLight bacterial viability kit L13152; Molecular Probes, Invitrogen), demonstrating that both bacteriophages were able to penetrate the biofilm and to produce the lysis of serotype b but not serotype c (Fig. 2). Furthermore, both phages were able to propagate in the infected biofilms (not shown).

Aabϕ01 and Aabϕ01-1 specifically infect *A. actinomycetemcomitans*; therefore, a possible clinical use of these phages would not affect the indigenous microbiota. In addition, they are highly selective in infection of serotype b but not serotype c. Serotype b is frequently associated with aggressive periodontitis (2, 13, 20), although differences in serotype distribution have been shown within the world population (10, 25), so the use of Aabϕ01 and Aabϕ01-1 against aggressive periodontitis, although possible, would not be universal. Despite this disadvantage, Aabϕ01 could be used as a rapid laboratory method to distinguish between serotype b and serotype c.

Our findings constitute the first report of an *in vitro* evaluation of a phage for *A. actinomycetemcomitans* to develop a therapy against the microorganism within its biofilm. Our results, then, show the identification of a novel bacteriophage for *A. actinomycetemcomitans* with the ability to eliminate the bacteria within its biofilm, a promising result of our search for a therapy helpful for the elimination of bacteria resistant to antibiotics and bacteria present in infectious biofilms. More studies involving phage therapy in animals and humans are necessary to determine the effectiveness of this alternative against aggressive periodontitis. Our laboratory is currently working on a comprehensive characterization of both Aabϕ01 and Aabϕ01-1 to reach a better understanding of their potentials as biocontrol agents.

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